Appl Microbiol Biotechnol (2001) 57:117–123
DOI 10.1007/s002530100780

ORIGINAL PAPER

K. P. Fayad · A.-M. Simao-Beaunoir · A. Gauthier
C. Leclerc · H. Mamady · C. Beaulieu · R. Brzezinski

Purification and properties of a β-1,6-glucanase from *Streptomyces* sp. EF-14, an actinomycete antagonistic to *Phytophthora* spp.

Received: 3 April 2001 / Received revision: 12 June 2001 / Accepted: 30 June 2001 / Published online: 11 August 2001

Abstract Extracellular enzymes with glucanase activities are an important component of actinomycete-fungus antagonism. *Streptomyces* sp. EF-14 has been previously identified as one of the most potent antagonists of *Phytophthora* spp. A β-1,6-glucanase (EC 3.2.1.75; glucan endo-1,6-β-glucosidase) was purified by four chromatographic steps from the culture supernatant of strain EF-14 grown on a medium with lyophilized cells of *Candida utilis* as main nutrient source. The glucanase level in this medium followed a characteristic pattern in which the rise of β-1,6-glucanase activity always preceded that of β-1,3-glucanase. The molecular mass of the enzyme was estimated to be 65 kDa and the pI approximately 5.5. It hydrolyzed pustulan by an endo-mechanism generating gentiobiose and glucose as final products. Laminarin was not hydrolyzed indicating that the enzyme does not recognize β-1,6-links flanked by β-1,3-links. No significant clearing of yeast cell walls in liquid suspensions or in agar plates was observed indicating that this β-1,6-glucanase is a non-lytic enzyme. This is the first β-1,6-glucanase characterized from an actinomycete.

Introduction

Among glucan-type polymers in fungal cell walls, those composed of monomers linked by β-1,3 or β-1,4-glucosidic links are predominant. Often, these glucans possess branches beginning with a β-1,6-glucosidic link. In some fungi, glucan chains made exclusively of β-1,6-linked residues can be found. For instance, various oomycetes belonging to the genus *Phytophthora* have two major glucan components in their cell wall: a linear, cellulose-like β-1,4-glucan and a highly branched β-1,3-glucan with branches starting with β-1,6 links and formed by an average number of four residues joined by β-1,3 links (Bartnicki-Garcia and Wang 1983). In the cell wall of *Candida albicans*, at least three distinct glucan fractions can be found: a highly branched β-1,6-glucan, a highly branched β-1,3-glucan, and a mixed β-1,3–β-1,6-glucan complexed to chitin (through a 1→6 link from a glucose residue from the mixed glucan to an *N*-acetyl-glucosamine residue of chitin) (Reiss et al. 1992). In common yeast *S. cerevisiae*, the β-1,6-glucan is found in linking chains between the other components of the cell wall, such as β-1,3-glucans, mannoproteins, and chitin (Kollár et al. 1997).

Total degradation of such fungal cell wall components requires the participation of enzymes having the capacity to hydrolyze the β-1,6 linkages in various molecular configurations (linear polymers, branched polymers, oligomeric molecules). Such enzymes have been detected in several bacteria and fungi but not in plants.

However, among the various glucan-hydrolyzing enzymes, the β-1,6-glucanases are still the least characterized group. Work on β-1,6-glucanases has been reviewed by Bielecki and Galas (1991) and by Pitson et al. (1996), who also presented an attempt at β-1,6-glucanase classification. The primary sequence of a β-1,6-glucanase has been determined only for an enzyme from *Trichoderma harzianum* (Lora et al. 1995).

The actinomycete *Streptomyces* sp. EF14 was isolated in our laboratory from the rhizosphere of potato (Faucher et al. 1992). During a screening program focused on the identification of strains antagonistic to *Phytophthora fragariae* var. *rubi* (the causal agent of raspberry root rot), EF14 raised further interest as one of the most efficient antagonists (Valois et al. 1996). The strain was able to lyse the mycelium of this pathogen in vitro as well as in vivo and was shown to produce a battery of glucanases, among which was a β-1,6-glucanase (Valois et al. 1996; Toussaint et al. 1997). In the present work, we purified and characterized a β-1,6-glucanase from this microorganism.

K.P. Fayad · A.-M. Simao-Beaunoir · A. Gauthier · C. Leclerc
H. Mamady · C. Beaulieu · R. Brzezinski (✉)
Centre d'Étude et de Valorisation de la Diversité Microbienne,
Département de Biologie, Faculté des Sciences,
Université de Sherbrooke, 2500, boul. de l'Université,
Sherbrooke (Québec) J1K 2R1 Canada
e-mail: rbrzezin@courrier.usherb.ca
Tel.: +1-819-821-8000 ext. 1077, Fax: +1-819-821-8049

Materials and methods

Microorganism and growth conditions

Streptomyces sp. EF-14 (ATCC BAA-282) was propagated at 30 °C on YME medium (0.4% glucose, 1% malt extract, and 0.4% yeast extract). On YME-agar medium, abundant sporulation was observed after 7–10 days of growth. The spores used for inoculation of glucanase production medium were collected from 15- to 20-day-old plates.

For β-1,6-glucanase production, a heavy inoculum of freshly harvested spores was introduced into 30 ml of YME medium. After 12–15 h of culture at 30 °C with shaking (350 rpm), the spores developed into very small mycelial pellets (accompanied by an increase in the optical density at 595 nm by 0.10–0.12 units). In order to avoid the rapid lysis of mycelium often observed at this stage, the culture was refreshed by transferring 2.5 ml of mycelial suspension into 250 ml of YME medium. This culture was further incubated for 9–10 h. Then, the culture was centrifuged at 3,000 rpm and the pellet was suspended in a few ml of sterile 0.85% NaCl. This suspension was used to inoculate 2 l of glucanase production medium prepared as follows: lyophilized cells of *Candida utilis* (Sigma), 10 g (dry weight) were suspended in 1,800 ml of distilled water, autoclaved, and then combined with 200 ml of sterile salt solution containing (per liter): $(NH_4)_2SO_4$, 10 g; K_2HPO_4, 5 g; $MgSO_4 \cdot 7H_2O$, 2 g; $FeSO_4 \cdot 7H_2O$, 100 mg. The culture was incubated for 12–15 h at 30 °C with vigorous shaking.

Enzyme assay and protein determination

β-1,6-Glucanase activity was determined by measuring the release of reducing sugars from pustulan (from *Umbillicaria papullosa*, Calbiochem). Before its use as substrate for enzymatic assay, the pustulan was reduced with borohydride as described (Schep et al. 1984), except that the ion-exchange step on Dowex resin was omitted and the ethanol precipitation step was repeated three times. The reduced pustulan (5 mg/ml) was dissolved in 100 mM Na-acetate buffer, pH 5.5, with heating; the solution is referred to as buffer-substrate (BS) solution.

The standard assay (0.5 ml) contained 490 µl BS and 10 µl of appropriately diluted enzyme sample. After 10 min of incubation at 50 °C, the reaction was stopped by the addition of 1 ml of *p*-hydroxybenzoic acid hydrazide reagent (Lever 1973) as modified by Schep et al. (1984). For the determination of reducing sugars, this mixture was boiled for 10 min, chilled in cold water, and the optical density read at 405 nm. Standards of glucose (0.1–0.4 mM) were also included. One unit of β-1,6-glucanase activity was defined as the amount of enzyme that released 1 µmol of glucose equivalent per min under the assay conditions.

Protein concentration was determined with the Coomassie blue assay (Bradford 1976) using bovine serum albumin (BSA) (Sigma) as standard.

β-1,6-Glucanase purification

Step 1: ethanol precipitation

The culture supernatant was supplemented with 2.5 mM EDTA and proteins were precipitated overnight at 4 °C with 3.5 volumes of ice-cold ethanol. After decantation, the pellet was recovered by low-speed centrifugation and resuspended in a minimal volume of 50 mM Na-acetate buffer, pH 4.4, with 2.5 mM EDTA (buffer A).

Step 2: ion-exchange chromatography

The product obtained from ethanol precipitation was loaded onto a 9×2.6-cm column with Macro-Prep High S (BioRad) resin equilibrated with buffer A. After loading, the column was washed with 100 ml of buffer A, and the proteins were eluted with 400 ml of a linear NaCl gradient (0–500 mM) in buffer A. The glucanase-containing fractions (58 ml) were diluted 10× with buffer A and loaded on a small (3×2.6 cm) column containing the same resin. The glucanase was recovered in a concentrated form by elution with 300 mM NaCl in buffer A.

Step 3: gel filtration

The concentrated pool was applied to a BioGel A-0.5m (BioRad) column (95×1.6 cm) equilibrated with 100 mM Na-acetate buffer, pH 5.5 (buffer B) supplemented with 2.5 mM EDTA and was eluted with the same buffer at a flow rate of 15 ml/h. The active fractions were pooled.

Step 4: size-exclusion HPLC

One ml of enzyme sample from step 3 was injected onto two connected in-series BioSil TSK-125 (BioRad) columns (600×7.5 mm) equilibrated with buffer B. Proteins were eluted with the same buffer at a flow rate of 1 ml/min. The active fractions were pooled.

Step 5: ion-exchange HPLC

The enzyme sample recovered from step 4 was acidified to pH 3.9 with acetic acid and diluted 2× with distilled water. Fifteen ml of this solution were injected onto a polysulfoethyl aspartamide (Nest Group, Southborough, Mass.) column (4.6×100 mm) equilibrated with 50 mM Na-acetate buffer, pH 3.9 (buffer C) at a flow rate of 1 ml/min. The column was washed with 5 ml of buffer C and the proteins were eluted with a linear NaCl gradient (0–500 mM) in buffer C. The active fractions were pooled.

Steps 1, 2, and 3 and the dialysis in step 5 were done at 4 °C. Steps 4, 5, and 6 were done at room temperature. Enzyme solutions were stored at 4 °C.

Lytic activity on yeast cell walls

Lytic activity was monitored using the turbidometric assay (Rombouts and Phaff 1976) modified as follows: 2 g of a commercial preparation of cell walls of *S. cerevisiae* (Lallemand, Montréal) were suspended in 10 ml of 50 mM Na-acetate buffer and dispersed by ultrasonication for 10 min. The suspension was then centrifuged (800 rpm, 10 min) and the pellet was washed three times with 5 ml of 10% sucrose and once with distilled water. The pellet was finally suspended in 15 ml of distilled water and stabilized with 0.02% sodium azide.

To assay lytic activity, 100 µl of yeast cell wall suspension were combined with 500 µl of Na-acetate buffer, pH 5.5, 300 µl of distilled water, and 100 µl of appropriately diluted enzyme sample and incubated for 18 h at 30 °C with mild agitation. Absorbance was measured at 450 nm (OD_{450}) in 1-cm cuvettes. The lytic activity (Rombouts and Phaff 1976) was calculated as the turbidometric index T_i=(OD_{450} at t=0/OD_{450} at t=18 h)–1. The assay was semi-quantitative, as the value of T_i did not increase linearly with the enzyme amount.

Other enzyme studies

All of the experiments described in this section were done in triplicate. The enzyme activity was tested on several polymeric or oligomeric carbohydrates dissolved or suspended in 50 mM Na-acetate buffer, pH 5.5, at a final concentration of 2.5 mg/ml (except for chitosan, which was dissolved at 0.8 mg/ml). The reaction products were detected as reducing sugars. Starch (soluble, from potato), xylan from oat spelts, carboxymethylcellulose, chitin (purified, from crab shells), chitosan (from crab shells, 80% deacetylated), laminarin (from *Laminaria digitata*), and gentiobiose

were obtained from Sigma. Pachyman (from *Poria cocos*) was obtained from Calbiochem (La Jolla, Calif.). Microcristalline cellulose was from FMC (Philadelphia, Pa.).

To determine the temperature optimum, enzyme activity was assayed over a temperature range of 22–70 °C for 10 min. The temperature stability of the glucanase was measured by preincubation of the enzyme in the absence of substrate for various times at a given temperature and assay of the residual activity by the standard assay (10 min at 50 °C).

SDS-PAGE was carried out using polyacrylamide gels of 5% and of 8% as stacking and separating gels, respectively. High-molecular-mass standard proteins (Bio-Rad) were used for molecular mass determination. Proteins were detected by staining the gels with Coomassie blue R-250.

Ampholine PAG plates with a pH range from 3.5 to 9.5 (Pharmacia Biotech) were used according to the manufacturer's specifications to determine the pI. The ampholine PAG-plate was stained with Coomassie blue R-250.

To determine pH optimum, the enzyme was assayed in 50 mM Na-citrate buffers covering a pH range from 4.0 to 6.0, or in 50 mM Tris-maleate buffers covering a range of 5.5 to 8.5; 2.5 mg/ml of pustulan served as substrate.

Thin-layer chromatography analysis of hydrolysis products

Pustulan or laminarin (dissolved at 2.5 mg/ml in 10 mM Na-acetate buffer, pH 5.5) was digested at 37 °C for various times with the purified β-1,6-glucanase and the resulting digests were heated for 10 min in boiling water bath to inactivate the enzyme, then concentrated by evaporation. Aliquots (25 µl, spotted in 5-µl portions) were applied on a silica gel thin-layer chromatography plate (Whatman AL SIL G/UV). Chromatograms were developed in a solvent system consisting of *n*-butanol, ethanol, and water in a 5:3:2 (v/v) proportion. The digestion products were revealed with a 95% ethanol-sulfuric acid-anisaldehyde (9:0.5:0.5) reagent by heating for 5 min. at 105 °C. Standards of glucose (4 mg/ml) and gentiobiose (4 mg/ml) were spotted in 5-µl portions.

Amino acid sequencing methods

The purified β-1,6-glucanase was transferred onto a PVDF membrane (Immobilon P, Millipore) and its N-terminal sequence was determined using the Applied Biosystems 473 A protein sequencer. To obtain an internal sequence of the protein, the β-1,6-glucanase was digested with trypsin and the resulting peptide fragment were purified by HPLC. The N-terminal portion of a purified peptide fragment was then sequenced. These experiments were performed by The Service Protéomique de l'est du Québec (Ste-Foy, Canada).

Results

Enzyme production

β-1,6-Glucanase was routinely produced on a complex medium containing a 0.5% suspension of lyophilized *Candida utilis* cells as main nutrient source. This substrate was chosen because of its commercial availability and also because the cell wall of another member of the genus *Candida* was shown to be rich in β-1,6-glucans, potential inducers for glucanase production (Reiss et al. 1992). Attempts to produce the enzyme on more defined media (such as a minimal medium with pustulan or gentiobiose as C source) resulted in low levels of glucanase. The enzyme was also rather poorly produced on media containing a cell wall preparation from *S. cerevisiae*. Also, media including higher percentages of *C. utilis* lyophilisate gave lower levels of glucanase production. By contrast, glucanase was efficiently produced in media containing 0.5% (dry weight basis) freshly grown cells of *C. utilis* or *C. tropicalis* (autoclaved but not lyophilized) (data not shown).

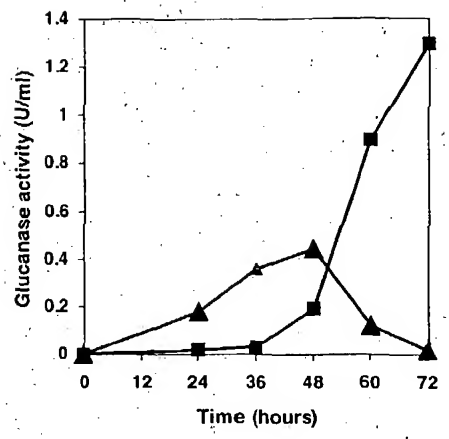

Fig. 1 Time-course of glucanase production after inoculation of *Streptomyces* sp. EF-14 in a medium containing lyophilized *Candida utilis* cells as nutrient source. ▲ β-1,6-glucanase, ■ β-1,3-glucanase

β-1,6-Glucanase production followed a characteristic pattern in this medium, the maximum of activity always preceding the increase in β-1,3-glucanase activity (Fig. 1). This could indicate that the hydrolysis products liberated from the fungal cell walls by the β-1,6-glucanase could facilitate the induction of β-1,3-glucanase expression.

Enzyme purification

The enzyme has been purified to apparent homogeneity (as judged from SDS-PAGE and from N-terminal sequencing) by a procedure involving one precipitation step and four chromatographic steps (Table 1). A 20-fold purification has been achieved with a 3.5% recovery of the β-1,6-glucanase activity.

Often, a brownish compound co-precipitated with the proteins during the ethanol precipitation step. This pigmented compound was still perceptible after the ion-exchange chromatography step but was eliminated after the size-exclusion chromatography step on Bio-Gel P-100. The same step was sufficient to separate the β-1,6-glucanase from the contaminating β-1,3-glucanase, which was eluted later from the column. It is worth notice that the first attempts to purify the enzyme by a size-exclusion chromatography step using a dextran-based Sephacryl S-200 HR resin were unsuccessful due to a strong affinity of the enzyme for the column bed. A similar effect has been observed (De la Cruz et al. 1995, 1999) during the characterization of endo-β-1,6-glucanases from *Trichoderma harzianum*.

Table 1 Purification of the β-1,6-glucanase

Purification step	Total activity (units)	Total proteins (mg)	Specific activity (U/mg protein)	Yield (%)	Purification factor
Culture filtrate	540	43.5	12.4	100	1
Ethanol precipitation	314	21.6	14.5	58.1	1.2
Bio-Gel P-100 size-exclusion chromatography	252	6.5	38.8	46.7	3.1
S-Sepharose fast-flow cation-exchange chromatography	169	1.5	111	31.2	9.0
TSK gel size-exclusion HPLC	43	0.25	172	8	13.9
Polysulfbethyl A cation-exchange HPLC	18.8	0.075	251	3.5	20.2

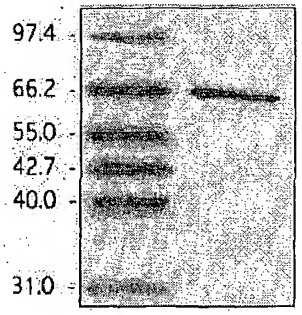

Fig. 2 Determination of molecular mass and assay of purity of β-1,6-glucanase by SDS-PAGE electrophoresis. *Left lane* Molecular mass markers, *right lane* purified β-1,6-glucanase

Properties of the β-1,6-glucanase

The molecular mass of the enzyme, determined by SDS-PAGE, was 65 kDa (Fig. 2). It corresponded to the mass calculated from size-exclusion HPLC column elution (step 4 of the purification procedure), indicating that the enzyme is a monomeric protein. The isoelectric point, determinated by isoelectric focusing, was 5.5 (not shown).

The optimal temperature for activity was determined by varying the incubation temperature at pH 5.5 in 10-min reactions. The enzyme was most active at 55 °C (Fig. 3a). In the standard assay (50 °C), the reaction progressed linearly for at least 2 h, indicating that the enzyme was stable at this temperature in the presence of pustulan (not shown). However, when preincubated in the absence of substrate and then tested for residual activity, the enzyme was rather unstable at temperatures higher than 45 °C (Fig. 3b) and its thermostability could not be significantly improved by the addition of BSA (0.1 mg/ml) (not shown). Thus, the presence of pustulan stabilizes the enzyme in standard assay conditions.

The enzyme displayed maximal activity at pH 5.5 and retained at least 80% activity at pH 4.5–6.5. (not shown). Kinetic analysis yielded an apparent K_m value of 0.19 mg/ml (with pustulan substrate) and a V_{max} of 284 U per mg protein. Activity was not affected by the presence of 1–20 mM D-glucono-1,5-lactone.

The N-terminal sequence as well as an internal sequence of the protein were determined as ADPTA-QVWVTTPDGA and TEDYQAYADYLVDT, respectively. A BlastP search failed to detect significant homology of these sequences with known proteins.

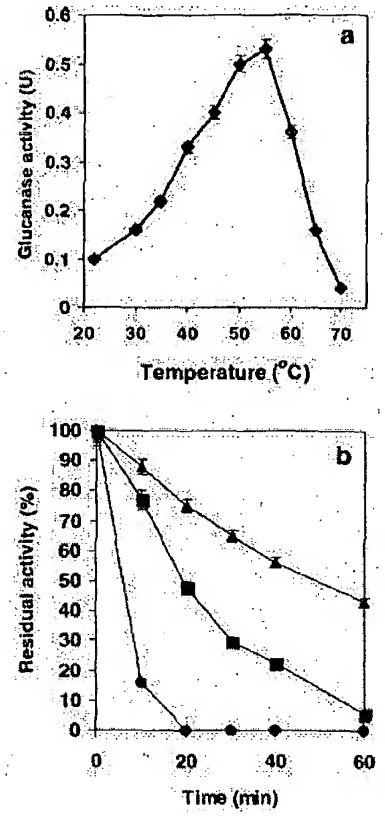

Fig. 3 **a** Effect of temperature on the activity of β-1,6-glucanase: 0.65 µg of glucanase were incubated with pustulan substrate for 10 min at 50 °C in 50 mM Na-acetate buffer, pH 5.5, at the temperature indicated, and the activity was measured by the release of reducing sugars. **b** Effect of temperature on the stability of β-1,6-glucanase in the absence of substrate. The enzyme was incubated for various times at the temperatures indicated in 50 mM Na-acetate buffer and the residual activity was measured by the standard assay. ▲ 45 °C, ■, 50 °C, ● 55 °C

Substrate specificity and action pattern

The substrate specificity of the purified β-1,6-glucanase was assayed against a variety of polysaccharides and oligosaccharides. (Table 2). No activity was detected against laminarin, a β-1,3-glucan, confirming that the purification procedure allowed the elimination of β-1,3-

Table 2 Substrate specificity of the purified β-1,6-glucanase

Substrate	Relative activity (%)
Pustulan	100
Starch	8
Xylan	12
Cellulose, microcrystalime	<0.1
Carboxymethylcellulose	<0.1
Chitin	<0.1
Chitosan	3
Pachyman	0.1
Laminarin	<0.1
Centiobiose	<0.1

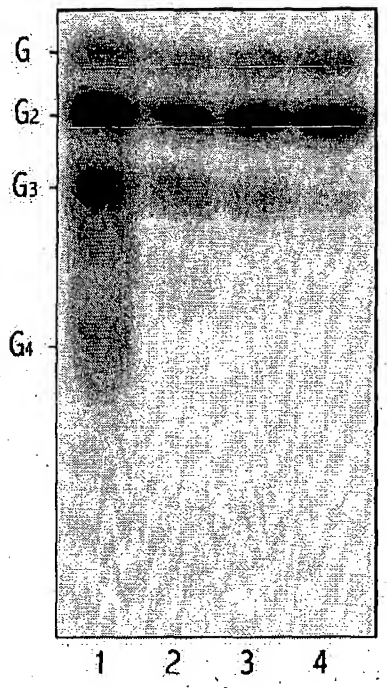

Fig. 4 Analysis of hydrolysis products by thin-layer chromatography. *Lane 1* Product generated after 24 h of incubation of pustulan (2.5 mg/ml) in 50 mM acetate-buffer with 10 mU of purified β-1,6-glucanase at 37 °C. *Lanes 2–4* Product obtained after addition of a fresh portion of 10 mU of glucanase to the hydrolysate shown in *lane* 1 and further incubation for 3 h (*lane 2*), 6 h (*lane 3*) or 24 h (*lane 4*). *G* Glucose, G_2–G_4 gentiobiose to gentiotetraose

glucanase activity. Low but significant activity was observed against xylan, starch, and chitosan. At present, we cannot establish if the observed activity resulted from the hydrolysis of the main substrate or if it derived from impurities present in the analyzed batches of these substrates.

Cell walls from *S. cerevisiae* were treated with 0.2 units of purified β-1,6-glucanase to detect a possible lytic activity. After 18 h of incubation, the turbidometric index was established at 0.17. This should be compared with the value of 1.08 obtained when the same cell-wall preparation was incubated with the EF-14 culture supernatant (100 µl) containing only 0.02 units of β-1,6-glucanase but also several other enzymatic activities (Valois et al. 1996). Following the criteria adopted by Rombouts and Phaff (1976), we concluded that the purified β-1,6-glucanase is a non-lytic enzyme, i.e. it is not capable by itself of causing significant lysis of yeast cell walls. This was confirmed by tests on agar plates containing an opaque yeast-cell-wall suspension: no perceptible clearing was observed when the β-1,6-glucanase was spotted onto the plate and incubated at 30 °C for 5 days. In contrast, clearing was observed after only 3–4 h of incubation when the same plates were treated with the EF-14 culture supernatant (not shown).

Using pustulan as substrate, the hydrolysis products were detected by thin-layer chromatography. Oligomers of four units were the main digestion product appearing after a 2-h reaction under the conditions previously described (data not shown). After a 24-h reaction, trimers, dimers, and monomers were the prevalent digest products (Fig. 4, lane 1). When the products of a 24-h digest were supplemented with increasing amounts of fresh enzyme and further incubated for several hours, a shift in the distribution was observed, with a decrease in the intensity of tetramers and trimers and a corresponding increase of dimers and monomers (Fig. 4, lanes 2–4). No release of sugars was observed in the absence of active enzyme.

Discussion

In previous studies (Valois et al. 1996; Toussaint et al. 1997), *Streptomyces* sp. EF-14 was characterized and was shown to exert a suppressive effect against root-rot diseases caused by *Phytophthora* spp. *Streptomyces* sp. EF-14 produces extracellular enzymes, including β-1,3-, β-1,4-, and β-1,6-glucanases which enable the strain to lyse *Phytophthora* cells. However, to establish the significance of glucanolytic enzymes in disease control, a wider knowledge about their properties is necessary. In this study, we characterized a β-1,6-glucanase, a type of enzyme that has not been purified previously from an actinomycete.

Recent work on β-1,6-glucanases has been mostly dedicated to enzymes from fungi. To our best knowledge, only four enzymes have been characterized in bacteria: two endoglucanases from *Bacillus circulans* (Rombouts and Phaff 1976, Rombouts et al. 1978), an endoglucanase from *Acinetobacter* sp. (Katohda et al. 1979), and a glucosidase from *Flavobacterium* (Sano et al. 1975). The exo-acting enzyme from *Flavobacterium* liberates glucose as the main product and acts efficiently on gentiobiose. The endo-acting enzymes can be roughly subdivided into "lytic" and "non-lytic" ones. The lytic endo-β-1,6-glucanase from *B. circulans* (Rombouts and Phaff 1976) generates gentiobiose and gentiotriose as final hydrolysis products (it does not hydrolyze gentiobiose) and has a relatively high activity against laminarin. The non-lytic endo-β-1,6-glucanases (Rombouts et al. 1976; Katohda et al. 1979) generate

Table 3 Heterogeneity of endo-β-1,6-glucanases. *Nd* Not done, *0.5% of the activity against pustulan

Organism	*Trichaderma harzianum* (BGN 16.1 glucanase)	*Trichoderma harzianum* (BGN 16.2 glucanase)	*Acremonium persicinum*	*Bacillus circulans* WL-12	*Streptomyces* sp. EF-14
Clearing of yeast cell walls	No	Yes	Nd	No	No
Release of glucose as final product	Yes	No	No	Yes	Yes
Gentiobiose-splitting activity	No	No	Low*	No	No
Ability to hydrolyze laminarin	Yes	No	Yes	No	No
Molecular mass (kDa)	51	43	42.7	52	65
Isoelectric point	7.4–7.7	5.8	4.9	8.2–8.3	5.5
Reference	De la Cruz and Llobell (1999)	De la Cruz et al. (1995)	Pitson et al. (1996)	Rombouts et al. (1978)	This work

glucose and gentiobiose as final hydrolysis products (again, they do not hydrolyze gentiobiose) and show no or very little activity against laminarin.

Many of the investigated fungal enzymes can be classified into the same two categories. Among recently studied enzymes, the enzyme from *Acremonium persicinum* (Pitson et al. 1996) appears to be an endo-acting β-1,6-glucanase, generating gentiobiose and gentiotriose as final products and showing very significant activity on laminarin; thus, this enzyme is similar to the lytic β-1,6-glucanase from *B. circulans* (Rombouts and Phaff 1976). Fungal enzymes similar to the bacterial non-lytic endo-β-1,6-glucanases have been characterized in *Penicillium brefeldianum* (Schep et al. 1984) and *Neurospora crassa* (Hiura et al. 1987).

The β-1,6-glucanase from *Streptomyces* sp. EF-14, characterized in the present work, acts by an endo-mechanism, as shown by the progressive release of oligomeric products from a polymeric substrate (pustulan). The final hydrolysis products are gentiobiose (the main product) and glucose. Gentotriose appears to be the shortest substrate productively recognized by the enzyme. Further evidence for the endo-mechanism is given by the fact that the enzyme is not inhibited by D-glucono-1,5-lactone and does not generate glucose from gentiobiose even after very long incubation periods with high enzyme loads (data not shown).

The β-1,6-glucanase from *Streptomyces* sp. EF-14 has no measurable activity on laminarin. This substrate contains mostly β-1,3-links besides β-1,6-links (in a ratio of ~7:1) (Bull and Chesters 1963). Since it is unable to hydrolyze the β-1,6-links present in laminarin, the enzyme should not productively recognize such links if they are localized in the proximity of β-1,3 links. Possibly, for efficient substrate binding and/or catalytic activity, this enzyme needs a substrate with an uninterrupted stretch of a few β-1,6-links. By contrast, as cited earlier, some enzymes are able to hydrolyze both pustulan and laminarin – a finding that is usually interpreted as the ability to hydrolyze not only 1,6-β-linkages but also 1,3-β-linkages involving 6-substituted glucose residues (Rombouts et al. 1978; Pitson et al. 1996). For many β-1,6-glucanases, the ability or inability to hydrolyze laminarin is well correlated with their respective properties of being "lytic" or "non-lytic" against fungal cell walls. Among glucanases previously characterized, the one showing most similarity with our glucanase is the non-lytic β-1,6-glucanase from *B. circulans* (Rombouts et al. 1978). However, the two enzymes differ by their molecular masses and even more markedly by their isoelectric points (Table 3), making it unlikely that they are closely related. The β-1,6-glucanase from *Streptomyces* sp. EF-14 is a non-lytic enzyme, which is also supported by the fact that the enzyme causes insignificant clearing of *Phytophthora* and yeast cell walls, both in liquid suspension and in agar plates. This does not necessarily imply that the β-1,6-glucanase of EF-14 is of no importance in the biocontrol process. *Phytophthora* cell walls do not contain long chains of β-1,6 linked residues. The action of the β-1,6-glucanase alone would not be sufficient to cause significant clearing of cell walls. However, the hydrolysis of these links might facilitate the access of the β-1,3-glucanases to their substrate and could in this way contribute to the lysis of the pathogen in vivo.

The β-1,6-glucanases thus appear as a highly heterogeneous group of enzymes. Table 3 illustrates this heterogeneity with a few examples, including the results of the present work. As in the case of other glycoside hydrolases (Henrissat and Davies 1997), molecular sequence data will be essential to build a comprehensive classification of β-1,6-glucanases.

Acknowledgements This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada and from the Conseil de recherches en pêches et en agroalimentaire du Québec. The experiments presented in this work comply with current laws in Canada.

References

Bartnicki-Garcia S, Wang MC (1983) Biochemical aspects of morphogenesis in *Phytophthora*. In: Erwin DC, Bartnicki-Garcia S, Tsao PH (eds) *Phytophthora*: its biology, taxonomy, ecology and pathology. APS, St. Paul, Minnesota, pp 121–137

Bielecki S, Galas E (1991) Microbial β-glucanases different from cellulases. Crit Rev Biotechnol 10:275–304

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Bull AT, Chesters CGC (1963) The biochemistry of laminarin and the nature of laminarinase. In: Nord FF (ed) Advances in enzymology. Interscience, New York, pp 325–364

De la Cruz J, Llobell A (1999) Purification and properties of a basic endo-β-1,6-glucanase (BGN16.1) from the antagonistic fungus *Trichoderma harzianum*. Eur J Biochem 265:145–151

De la Cruz J, Pintor-Toro JA, Benítez T, Llobell A (1995) Purification and characterization of an endo-β-1,6-glucanase from *Trichoderma harzianum* that is related to its mycoparasitism. J Bacteriol 177:1864–1871

Faucher E, Savard T, Beaulieu C (1992) Characterization of actinomycetes isolated from common scab lesions on potato tubers. Can J Plant Pathol 14:197–202

Henrissat B, Davies G (1997) Structural and sequence-based classification of glycoside hydrolases. Curr Opin Struct Biol 7:637–644

Hiura N, Nakajima T, Matsuda K (1987) Purification and some properties of an endo-b-1,6-glucanase from *Neurospora crassa*. Agric Biol Chem 51:3315–3321

Katohda S, Suzuki F, Katsuki S, Sato T (1979) Purification and some properties of endo-β-1,6-glucanase from *Acinetobacter* sp. Agric Biol Chem 43:2029-2034

Kollár R, Reinhold BB, Petráková E, Yeh HJC, Ashwell G, Drgonováa J, Kapteyn JC, Klis FM, Cabib E (1997) Architecture of the yeast cell wall. β-1,6-glucan interconnects mannoproteins, β-1,3-glucan and chitin. J Biol Chem 272:17762–17775

Lever M (1973) Colorimetric and fluorometric carbohydrate determination with *p*-hydroxybenzoic acid hydrazide. Biochem Med 7:274–281

Lora JM, De la Cruz J, Llobell A, Benítez T, Pintor-Toro JA (1995) Molecular characterization and heterologous expression of an endo-β-1,6-glucanase gene from the mycoparasitic fungus *Trichoderma harzianum*. Mol Gen Genet 247:639–645

Pitson SM, Seviour RJ, McDougall BM, Stone BA, Sadek M (1996) Purification and characterization of an extracellular (1→6)-β-glucanase from the filamentous fungus *Acremonium persicinum*. Biochem J 316:841–846

Reiss E, Hearn VM, Poulain D, Shepherd MG (1992) Structure and function of the fungal cell wall. J Med Vet Mycol 30 (Suppl 1):143–156

Rombouts FM, Phaff HJ (1976) Lysis of yeast cell walls. Lytic β-(1→6)-glucanase from *Bacillus circulans* WL-12. Eur J Biochem 63:109–120

Rombouts FM, Fleet GH, Manners DJ, Phaff HJ (1978) Lysis of yeast cell-walls: non-lytic and lytic (1→6)-β-D-glucanases from *Bacillus circulans* WL-12. Carbohydr Res 64:237–249

Sano K, Amemura A, Harada T (1975) Purification and properties of a β-1,6-glucosidase from *Flavobacterium*. Biochim Biophys Acta 377:410–420

Schep GP, Shepherd MG, Sullivan PA (1984) Purification and properties of a β-1,6-glucanase from *Penicillium brefeldianum* Biochem J 223:707–714

Toussaint V, Valois D, Dodier M, Faucher E, Déry CV, Brzezinski R, Ruest L, Beaulieu C (1997) Characterization of actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the causal agent of raspberry root rot. Phytoprotection 78:43–51

Valois D, Fayad K, Barasubiye T, Garon M, Déry CV, Brzezinski R, Beaulieu C (1996) Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae var. rubi*, the causal agent of raspberry root rot. Appl Envir Microb 62:1630–1635